Kidney International, Vol. 45 (1994), pp. 1070–1076

K^+-sparing diuretic actions of trimethoprim: Inhibition of Na^+ channels in A6 distal nephron cells

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K^+ sparing diuretic actions of trimethoprim: Inhibition of Na^+ channels in A6 distal nephron cells. Hyperkalemia complicates trimethoprim-sulfamethoxazole (TMP-SMX) therapy in over 20% of HIV-infected patients. TMP is a heterocyclic weak base, similar to amiloride, a "K^+-sparing" diuretic and Na^+ channel blocker. Apical TMP is known to inhibit amiloride-sensitive short circuit current in A6 cells, a tissue culture model for mammalian cortical collecting tubule principal cells [1]. We used cell-attached patch clamp techniques to investigate the effect of TMP on the 4 pS, highly selective Na^+ channel in the apical membrane of A6 cells grown on permeable supports in the presence of 1.5 μM aldosterone. Baseline channel activity at resting membrane potential, measured as NP_o (N of channels $\times$ open probability), was 1.09 ± 0.50 ($N = 18$). NP_o (0.92 ± 0.38; $N = 9$) was unchanged when 10^{-3} M TMP was added to the basolateral bath for 30 minutes. However, apical exposure with pipettes containing 10^{-3} or 10^{-5} M TMP reduced $NP_o \approx$ tenfold (0.12 ± 0.08; $N = 7$ and 0.18 ± 0.14; $N = 12$, respectively). Kinetic analysis revealed the appearance of a new closed state after apical TMP treatment. Another group of A6 cells were pretreated with 10^{-3} M apical TMP for 30 minutes prior to patching with pipettes filled with TMP-free saline. NP_o progressively rose from 0.07 ± 0.09 to 0.87 ± 0.23 ($N = 5$) as the residual TMP was diluted within the pipette. Apical or basolateral pretreatment (30 min) with 10^{-3} M SMX did not change Na^+ channel activity. In conclusion, in A6 distal nephron cells: (1) TMP reversibly blocks highly selective Na^+ channels; (2) direct interaction with the outer channel pore is required since inhibition was observed with apical, but not basolateral TMP; (3) the SMX component of TMP-SMX preparations has no direct effect on Na^+ channel activity; (4) This K^+-sparing diuretic effect likely contributes to the hyperkalemia associated with TMP therapy in HIV-infected patients.

Drug-induced hyperkalemia has not been associated with conventional doses of the antimicrobial agent, trimethoprim-sulfamethoxazole (TMP-SMX) [2, 3]. However, in recent clinical studies hyperkalemia has been observed in 20 to 53% of HIV-infected patients receiving high dose TMP-SMX or TMP-dapsone for the treatment of the opportunistic infection, *Pneumocystis carinii* pneumonia [1, 4–7]. This hyperkalemia is associated with an inappropriate decrease in kaliuresis, but is not related to adrenal insufficiency, renal failure or tubulointerstitial nephritis. These results suggest that TMP may directly interfere with ion transport in the distal nephron.

Regulation of K^+ excretion takes place primarily in the cortical collecting tubule (CCT), and specifically, in principal cells [8–10]. The electrochemical driving force for K^+ secretion in this segment of the nephron is dependent on luminal Na^+ entry via apical Na^+ channels and on serosal K^+ uptake via the basolateral Na^+/K^+-ATPase pump [8–10].

Trimethoprim, a 2,4 diaminopyrimidine, and the "potassium-sparing" diuretics, amiloride and triamterene, are all heterocyclic weak bases which exist primarily in their protonated forms at normal urinary pH (Fig. 1). It is the protonated form of these diuretics which promotes natriuresis by inhibiting Na^+ reabsorption in the CCT [1]. Using patch clamp technology, we and other groups have shown that apical amiloride directly blocks highly selective Na^+ channels in both mammalian and amphibian distal nephron cells [11–13]. Several groups have postulated that TMP might have similar K^+-sparing diuretic actions and thus predispose patients to hyperkalemia [1, 5, 14].

A6 is a distal nephron cell line derived from *Xenopus laevis*, which provides an excellent model for mammalian CCT principal cells [13]. Recently, macroscopic measurements in A6 cell monolayers have shown inhibition of amiloride-sensitive, short circuit current (I_{sc}) by apical TMP with half maximal inhibition occurring at concentrations of 1.2×10^{-4} M [1]. The amiloride-sensitive component of I_{sc} is usually assumed to represent net transepithelial sodium transport. However, I_{sc} actually equals the algebraic sum of all currents across the apical membrane and recent studies in A6 cells indicate that other apical conductances besides Na^+ influence the measurement of I_{sc} [15, 16]. To clarify the mechanism for this TMP-induced electrophysiologic response at the level of individual ion channels, we applied cell-attached patch clamp techniques to A6 cells and studied the effect of TMP on highly selective, apical Na^+ channels.

Methods

Preparation of A6 distal nephron cell line cultures

The methods are similar to those described previously [1, 13]. Briefly, A6 cells (American Type Culture Collection, Rockville, Maryland, USA or a gift from Dr. N.K. Wills) were maintained in plastic tissue culture flasks at 26°C with 4% CO_2 in air. The modified culture medium consisted of Coon's F-12 medium, Leibovitz's L-15 medium, 0.6% penicillin/1.0% streptomycin,

Received for publication September 8, 1993
and in revised form November 3, 1993
Accepted for publication November 5, 1993

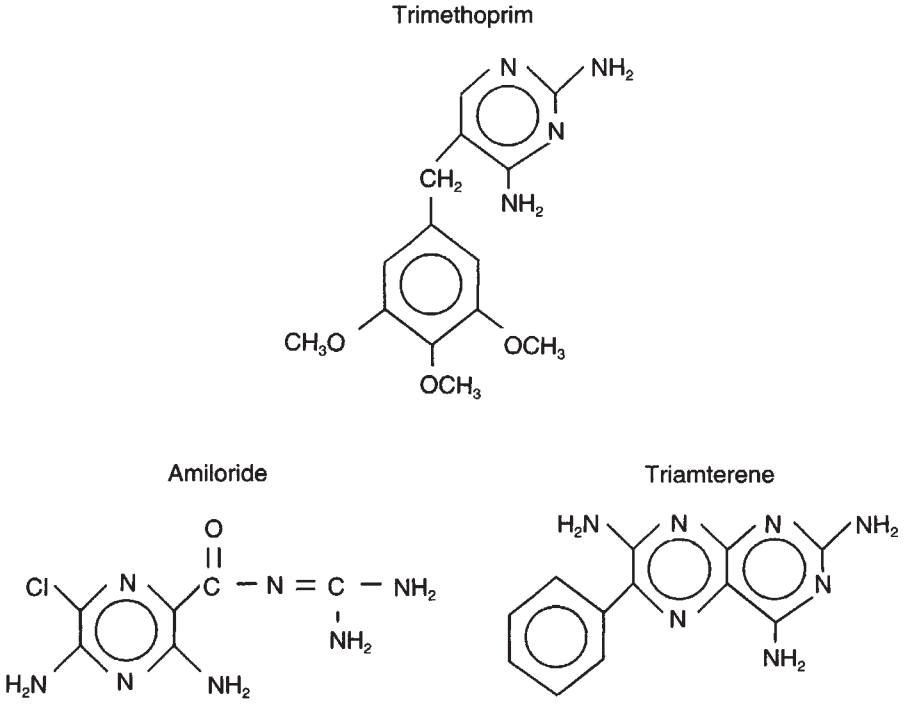

Fig. 1. *Chemical structures for trimethoprim (TMP) and the "K^+-sparing diuretics," amiloride and triamterene.*

10% (vol/vol) fetal bovine serum, 1.5 μM aldosterone, 1 mM glutamine and 25 mM $NaHCO_3$. A6 cell passages 71 to 85 were used for patch clamp experiments and passages 91 to 96 for transepithelial measurements. For patch clamp experiments, A6 cells were plated at confluent density on permeable, glutaraldehyde-fixed, collagen-coated Millipore-CM filters (Millipore Corp., Massachusetts, USA) attached to the bottoms of small lucite rings [8, 11]. This sided preparation allowed patch pipette access to the apical membrane, and separate control of the apical and basolateral bath compositions. For transepithelial experiments, A6 cells were seeded on collagen-coated polycarbonate filters (Costar, California, USA) at a density of 0.5 to 1.0 $\times 10^6$ cells/cm^2 [1].

Patch clamp recording and analysis

A6 distal nephron cells were visualized with Hoffman modulation optics (Modulation Optics Inc., New York, USA) mounted on a Nikon Diaphot-TMD inverted microscope as previously described [8, 17]. Patch pipette and extracellular bath solutions consisted of a physiologic amphibian saline containing (mM): 95 NaCl, 3.4 KCl, 0.8 $CaCl_2$, 0.8 $MgCl_2$, and 10 HEPES (pH 7.4). Experiments were performed at room temperature. Unitary channel events were measured using a List EPC-7 Patch Clamp (Medical Systems Corp., New York, USA), digitized by a DAS 601 Pulse Code Modulator (Dagan Corp., Minnesota, USA) and recorded on a SL-HF860D video cassette recorder (Sony Corp. of America, New Jersey, USA). Data were acquired using a 902LPF 8-pole Bessel filter (Frequency Devices Inc., Massachusetts, USA), TL-2 acquisition hardware and Axotape software (Axon Instruments Inc., California, USA). The convention for applied voltage to the apical membrane patch ($-V_{pipet}$) represents the voltage deflection from the patch potential (that is, the resting membrane potential for cell-attached patches) and is expressed as the potential of the cell interior with respect to the patch pipette interior (that is, negative voltage = hyperpolarization; positive voltage = depolarization). Inward current (pipette to cell) is represented as downward transitions in single channel records.

Analysis of data was performed on a 386SX computer (Mitsuba Southeast Inc., Georgia, USA) utilizing locally- and commercially-developed software [8, 11]. The total number of functional channels (N) in the patch were estimated by observing the number of peaks detected on current amplitude histograms. As a measure of channel activity, NP_o (number of channels times the open probability) was calculated.

$$NP_o = \sum_{n=0}^{N} \frac{n \cdot t_n}{T} \quad (1)$$

T was the total record time, n was the number of channels open and t_n was the record time during which n channels are open. Therefore, NP_o can be calculated without making assumptions about the total number of channels in a patch or the open probability of a single channel. All results are expressed as the mean ± SE.

Transpithelial measurements

A6 cell monolayers were transferred to a modified Ussing chamber and bathed in a Ringer's saline containing (mM): 100 NaCl, 4 KCl, 2.5 $NaHCO_3$, 1 KPO_4, 1 $CaCl_2$, 11 glucose, and 10 HEPES (pH 7.4) [1]. Electrical measurements were performed with a DVC-1000 voltage clamp (World Precision Instruments, Florida, USA). The short circuit current (I_{sc}) was allowed to stabilize before the addition of drug or vehicle. The amiloride-sensitive component of the short circuit current was determined

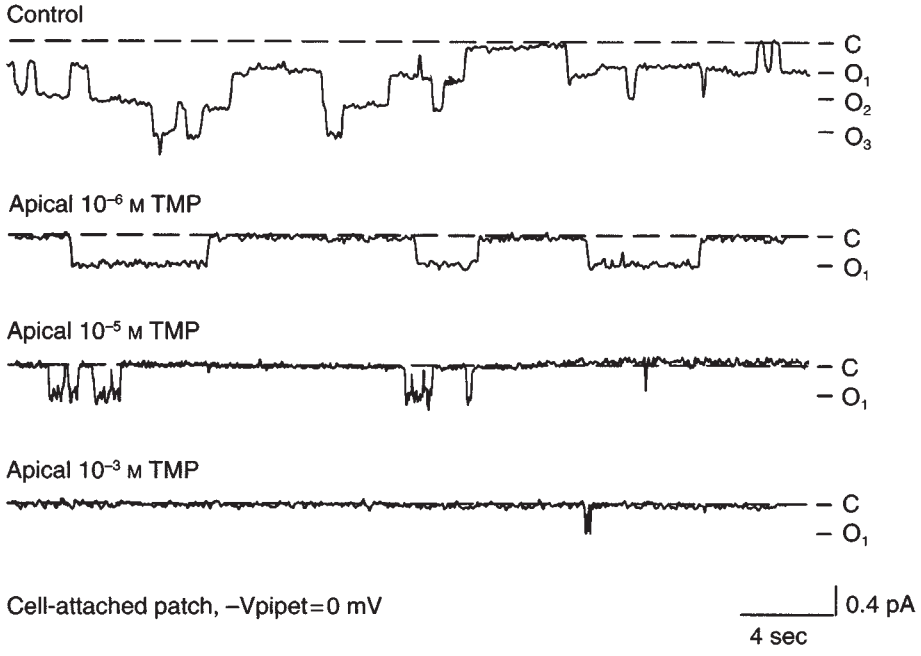

Fig. 2. *Effect of apical TMP on highly sensitive Na^+ channels.* Cell-attached patch recordings were made on A6 cells using pipettes backfilled with physiologic saline (control) or various concentration of TMP. Downward deflections indicate inward Na^+ current (pA) and "C" marks zero current level (closed state). Progressive inhibition of channel activity is seen with increasing apical TMP concentrations. $-V_{pipet}$ was 0 mV. Original corner frequency (Fc) was 1 KHz, sampling was at 2 KHz and additional software filtering was 200 Hz for all recordings.

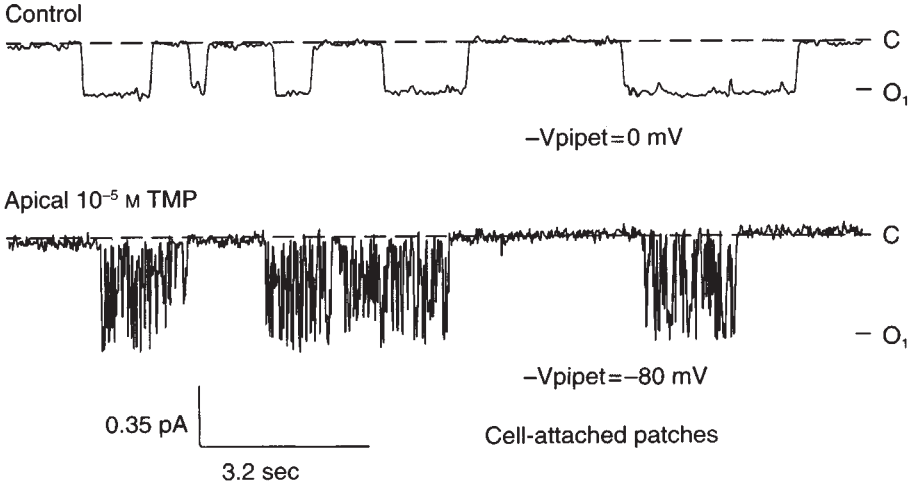

Fig. 3. *TMP-induced Na^+ channel "flickering."* Cell-attached patch showed control activity with typical long open and closed events durations (seconds). In the same patch frequent, brief transitions to the closed state appear after TMP diffuses to the channel pore through the 10^{-5} M TMP-backfilled pipette. This "flickering" behavior is better visualized at hyperpolarized potential ($-V_{pipet} = -80$ mV).

by adding 10^{-5} M amiloride to the luminal solution at the end of each experiment.

Chemicals

Trimethoprim, sulfamethoxazole and amiloride were purchased from Calbiochem Corp., or Sigma Chemical. Appropriate solvent vehicles were added to control baths and, by themselves, caused no change in Na^+ channel activity or I_{sc}.

Results

Apical trimethoprim inhibits highly selective Na^+ channel activity

Apical membrane cell-attached patches were established on A6 cells exposed to physiologic saline in both the apical and basolateral baths (Fig. 2). Control traces revealed spontaneous inward current events with long open and closed times which is characteristic of highly selective Na^+ channels under basal conditions (resting membrane potential, room temperature, physiologic extracellular ion composition, intact cell-attached configuration) [13]. Cell-attached patches were then made using patch pipettes filled with various concentrations of TMP (10^{-6} to 10^{-3} M). Progressive inhibition of single Na^+ channel activity was evident with exposure to increasing concentrations of apical TMP. With pipettes containing 10^{-5} M TMP, we observed rapid "flickering" between the open and closed states. This kinetic behavior was better illustrated at hyperpolarized potentials (Fig. 3). At still higher concentrations of apical TMP (10^{-3} M), Na^+ channel activity was almost completely abolished; only rare, brief open events were observed (Fig. 2).

As a measure of Na^+ channel activity, NP_o (number of channels times open probability) was calculated from five minutes of continuous cell-attached recording under various conditions (Fig. 4). Under basal conditions, control channel activity had a wide range of values with a mean NP_o of 1.09 ±

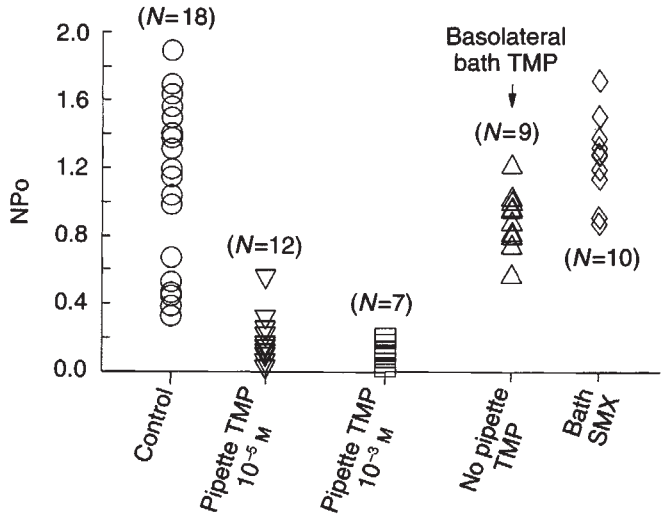

Fig. 4. *Na^+ channel activity after TMP exposure.* The ordinate represents the channel activity (NP_o) in the absence of TMP (○; N = 18), with 10^{-5} M (▽; N = 12) or 10^{-3} M (□; N = 7) TMP in the pipette solution only, and after exposure to 10^{-3} M TMP in the basolateral bath solution only for 30 minutes (△; N = 9). Channel activity was unaffected by preincubation of A6 cells with 10^{-3} SMX (◇) in either the apical (N = 5) or basolateral (N = 5) bath.

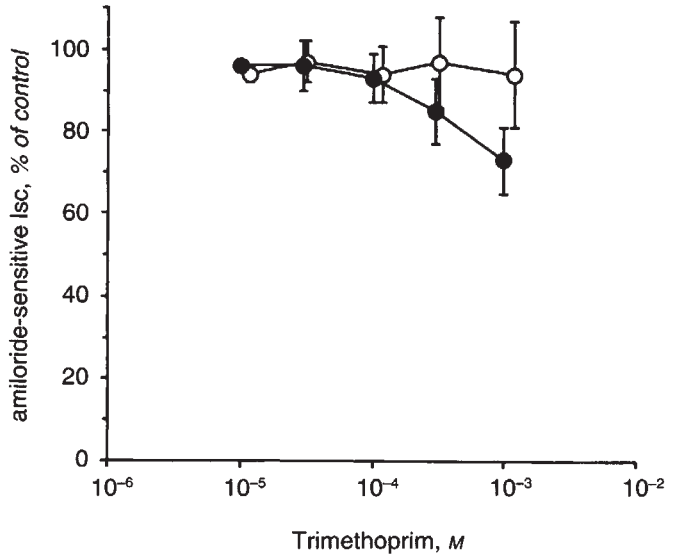

Fig. 6. *Effect of basolateral bath TMP on I_{sc} in A6 cell monolayers.* Symbols are: (○) control; (●) trimethoprim. The amiloride-sensitive component of I_{sc} is expressed as a percentage of total I_{sc}. The results are the mean ± SE values from six experiments.

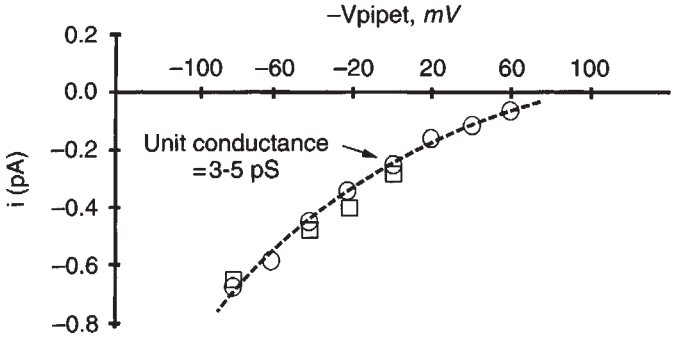

Fig. 5. *Na^+ channel voltage (I-V) relationship.* Data from cell-attached patches with pipettes containing physiologic saline (○) or 10^{-5} M TMP (□) yields similar I-V curves, consistent with a channel ion selectivity much higher for Na^+ than for K^+. Unitary conductance (3 to 5 pS) was calculated from the I-V curve slope near resting membrane potential.

0.50 (N = 18). When patch pipettes were backfilled with 10^{-5} M or 10^{-3} M TMP, mean NP_o decreased by 83% (0.18 ± 0.14; N = 12) and 89% (0.12 ± 0.08; N = 7), respectively.

Apical exposure of A6 cells to 10^{-5} TMP in the pipette solution did not alter or shift the Na^+ channel current-voltage relationship, indicating that TMP did not significantly change single channel conductance (3 to 5 pS) or reversal potential (Fig. 5).

Na^+ channel activity is not affected by SMX or basolateral TMP

Another set of A6 cells were pretreated for 30 minutes with 10^{-3} M TMP in the basolateral bath only. Na^+ channel activity with cell-attached patch pipettes containing TMP-free saline (mean NP_o = 0.92 ± 0.38; N = 9) was not statistically different from control patches (mean NP_o = 1.09 ± 0.50; N = 18; Fig. 4).

This lack of effect for basolateral TMP was confirmed by macroscopic measurements of amiloride-sensitive I_{sc} in A6 cell monolayers (Fig. 6).

Clinical preparations of TMP often contain sulfamethoxazole (SMX) [1, 4, 18]. However, pretreatment for 30 minutes with 10^{-3} M SMX alone in the apical (N = 5) or the basolateral bath (N = 5) had no effect on basal Na^+ channel activity in cell-attached patches (mean NP_o = 1.27 ± 0.41; Fig. 4).

Kinetics of apical TMP-induced Na^+ channel inhibition

Kinetic analysis was performed from time interval histograms generated from cell-attached recordings where the number of observed Na^+ channels were few (no recordings contained only a single channel; Fig. 7). Under control conditions, open time histograms were best fit by one exponential distribution representing one single open state with a mean open time of 705.8 ± 329.1 msec (N = 5). When 10^{-5} M TMP was present in the patch pipette, the open time interval histograms again revealed one exponential distribution. However, with 10^{-5} TMP in the pipette, the mean open time decreased 40-fold (17.2 ± 9.4 msec; N = 6). Under control conditions, the closed-time interval histograms were also best fit by one exponential with a mean closed time of 1.56 ± 0.52 sec (N = 5). With apical TMP present, a second exponential distribution appeared. The shorter mean closed time was 9.73 ± 4.05 msec and the longer mean closed time was 3.15 ± 1.29 sec (N = 6).

Data in Figure 8 show reversibility of the TMP-induced channel inhibition. A6 cell monolayers were preincubated with 10^{-3} M TMP in the apical bath for 30 minutes prior to applying the patch clamp. NP_o was then measured for the five minutes immediately following cell-attached patch formation with pipettes containing TMP-free saline (mean NP_o = 0.07 ± 0.09; N = 5). Mean NP_o was 0.53 ± 0.21 (N = 5) and 0.87 ± 0.23 (N = 5) over the 5 to 10 minute and 10 to 15 minute periods

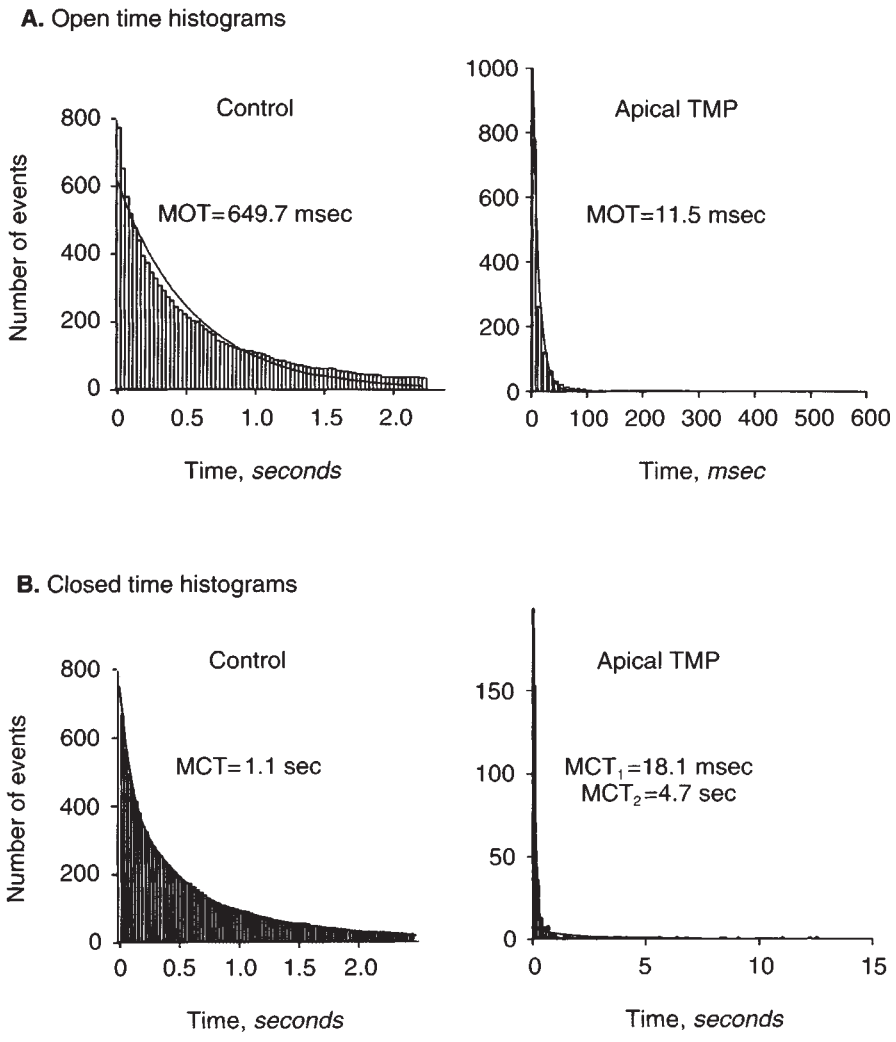

Fig. 7. *High selectivity Na^+ channel kinetics.* **(A)** Open time interval histogram. In the absence of TMP (Control), the histogram is best fit by one exponential distribution with a mean open time (MOT) of 649.7 miliseconds (left). With 10^{-5} M TMP in the pipettes, the histogram also shows one distribution with MOT decreasing to 11.5 miliseconds (right). **(B)** Closed time interval histogram. The control histogram is best fit by one exponential with a mean closed time (MCT) of 1.1 seconds (left). With 10^{-5} M TMP in the pipettes (right), the histogram again shows the usual long (seconds) mean closed time (MCT_2) distribution. In addition, a second exponential distribution with a shorter (miliseconds) MCT_1 appears. Histograms are generated from a typical single channel record. Mean open and closed time values in the text are averaged from several records.

following patch formation, respectively; Na^+ channel activity consistently rose in each cell-attached patch. These results suggest that the saline in the pipette dilutes the TMP interacting with the apical membrane surface.

Discussion

Patients infected with the human immunodeficiency virus have a predilection for opportunistic infections, the most common being *Pneumocystis carinii* (PCP) pneumonia [4, 6]. First-line therapy for severe cases of PCP pneumonia consists of high doses of parenterally administered TMP [4, 6]. The clinical course of HIV-infected patients receiving TMP-containing drugs, either TMP-SMX or TMP-dapsone, is often (20 to 53%) complicated by elevations in serum potassium level [1, 4, 5]. Studies show that the hyperkalemia can occur without the renal failure, adrenal insufficiency, hyporeninemic hypoaldosteronism or tubulointerstitial nephritis which often complicates HIV infection [1, 7, 19]. In the absence of such underlying diseases, the hyperkalemia resolves spontaneously after discontinuation of TMP therapy. Additionally, hyponatremia, increased natriuresis and decreased kaliuresis were observed in these TMP-treated patients, despite the presence of hyperkalemia [1, 7, 19]. Rat studies by Velazquez and associates [14] reveal that intravenous TMP inhibits renal K^+ secretion by 40% and increases renal Na^+ excretion by 46%. Distal tubule microperfusion experiments also revealed that intratubular TMP inhibits distal tubule K^+ secretion by 59% and depolarizes the lumen-negative transepithelial voltage by 66%. These findings suggest that TMP might interfere with distal nephron ion transport, the primary site for regulation of renal tubular K^+ excretion and Na^+ reabsorption [8–10].

However, the renal epithelial ion transport defect and the mechanism responsible for this TMP-induced decrease in K^+ excretion and Na^+ reabsorption has not been defined at a single channel level. The electrochemical driving force for K^+ secretion in the distal nephron is maintained by luminal Na^+ entry via apical Na^+ channels and serosal K^+ uptake via the basolateral Na^+/K^+-ATPase pump [8–10].

TMP inhibits Na^+ channel activity in A6 distal nephron cells

In the present study, we have demonstrated that apical, but not basolateral exposure to TMP inhibits the activity of amiloride-sensitive, highly selective Na^+ channels in the A6 distal nephron cell line. This side effect was confirmed in

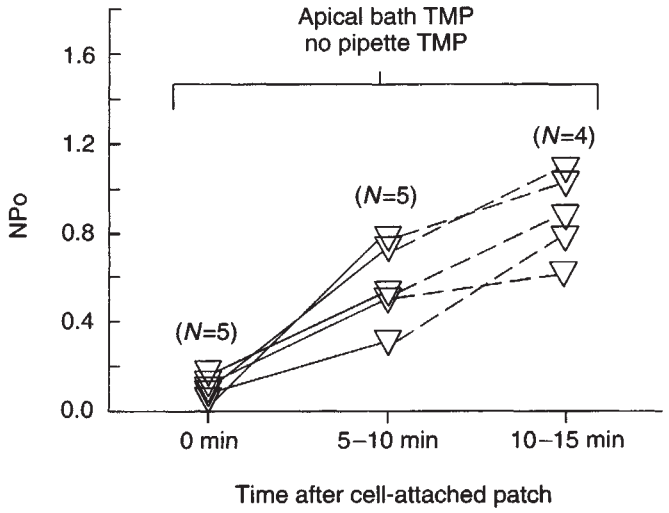

Fig. 8. *Reversibility of TMP-induced Na^+ channel block.* A6 cell monolayers were preincubated with 10^{-3} M TMP in the apical bath only for 30 minutes prior to patching. NP_o was then measured for the 5 minutes immediately following cell-attached patch formation with pipettes containing TMP-free saline (0 min). With the intrapipette saline diluting the TMP interacting with the apical membrane surface, Na^+ channel activity consistently rose over the 5 to 10 min and 10 to 15 min periods following patch formation. Lines connecting symbols represent values from the same cell-attached patch.

macroscopic I_{sc} measurements on A6 cell monolayers. We found that apical TMP concentrations of 10^{-5} to 10^{-3} M inhibited NP_o for Na^+ channels by 83 to 89% in cell-attached patches. The current-voltage relationship did not shift or alter with apical TMP exposure, indicating that TMP does not significantly affect single Na^+ channel conductance, ion selectivity or apical membrane potential.

In humans, TMP has a half-life of 8 to 12 hours and 80% is excreted unchanged into the urine [6]. TMP concentrations in the range of 10^{-4} to 10^{-3} M appear in the urine of patients receiving oral TMP at doses only half the concentration of the intravenous TMP doses used to treat HIV-infected patients with PCP pneumonia [1, 14, 18]. Therefore, clinically relevant urinary TMP concentrations are capable of significant distal nephron Na^+ channel inhibition.

Clinical preparations of TMP often contain sulfamethoxazole (SMX) [1, 4, 18]. However, we found that neither apical or basolateral exposure to SMX affected basal Na^+ channel activity. Consistent with our single Na^+ channel findings, Kleyman and associates [1] have also shown no effect of either apical or basolateral SMX on amiloride-sensitive Na^+ transport (I_{sc}) in A6 cells.

TMP acts by directly blocking apical Na^+ channels

TMP's inhibitory effects require direct luminal contact with the channel pore since basolateral TMP has no effect on basal Na^+ channel activity. Since the 2,4 diaminopyridine structure of TMP bears similarities to amiloride, we investigated whether TMP might also be acting as a direct Na^+ channel blocking agent [1].

Single channel records reveal that apical TMP induced new frequent, brief channel closings giving the Na^+ channel open events a flickering appearance and suggesting that channel kinetics were affected. Under basal conditions, time-interval histograms revealed one open state and one closed state, with both mean open and closed times lasting approximately a second. In addition to the usual longer closed times (seconds), a new distribution of shorter closed times (miliseconds) appears with exposure to apical TMP, the latter representing a new blocked state. The mean open time also decreases with apical TMP, reflecting the appearance of an additional pathway for transition out of the open state. We also demonstrated that the TMP-induced block of the Na^+ channel is rapidly reversed by dilution with TMP-free saline. The simplest kinetic scheme which fits our results involves reversible binding of the TMP molecule to the outer surface of open Na^+ channels:

$$\text{Closed} \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} \text{Open}$$

$$Na^+ \text{ channel blocker} \downarrow \text{Amiloride or TMP?}$$

$$\text{Closed} \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} \text{Open} \underset{k_{-2}}{\overset{k_2}{\rightleftharpoons}} \text{Blocked}$$

This TMP-induced blocking effect is similar to Na^+ channel protein interactions with apical amiloride [12, 13, 20].

Macroscopic measurements by Kleyman and associates [1] also reveal a dose-dependent inhibition of amiloride-sensitive I_{sc} in A6 cell monolayers by apical TMP with an IC_{50} of 1.2×10^{-4} M. While the amiloride-sensitive component of I_{sc} has been assumed to be a measure of net transepithelial sodium transport, I_{sc} actually equals the algebraic sum of all currents across the apical membrane. Recent studies in distal nephron cells indicate that other ion conductance pathways (Cl^-, K^+ and nonselective cation channels) besides Na^+ channels also exist in the apical membrane and influence the measurement of I_{sc} [8, 11, 15, 16, 21]. Amiloride-induced Na^+ channel inhibition results in hyperpolarization of the apical membrane potential, producing an electrical driving force favoring apical Cl^- secretion and cation reabsorption under short-circuit conditions. In addition, macroscopic studies demonstrate only that TMP inhibits total apical Na^+ current, but they do not reveal whether this effect is mediated by changing the electrochemical gradient (that is, Na^+/K^+-ATPase) for Na^+ reabsorption or single Na^+ channel properties (that is, unit conductance, number or kinetics). Finally, macroscopic studies have not revealed whether apical TMP affects the Na^+ channel directly or indirectly through some intracellular signalling cascade.

Therefore, to clarify the mechanism for this TMP-induced electrophysiologic response at the level of individual ion channels, we applied cell-attached patch clamp techniques to A6 cells and studied the effect of TMP on highly selective, apical Na^+ channels. Our patch clamp experiments show inhibition of single Na^+ channels at even lower doses than observed with I_{sc} measurements [1]; >80% inhibition of NP_o at intrapipette TMP concentrations of 10^{-5} M. This shifted dose response curve for TMP-induced inhibition of inward Na^+ current likely represents a reduction in feedback inhibition of apical Na^+ channels under I_{sc} conditions. We have demonstrated in A6 cells that there exists feedback inhibition of Na^+ channel open activity in cell-attached patches in response to increases in apical Na^+

entry [22, 23]. Apical Na^+ entry increases intracellular Na^+ and Ca^{2+} levels via basolateral Na^+/Ca^{2+} exchange, and leads to inhibition of Na^+ channel activity through the activation of apical-membrane bound protein kinase C. While only the Na^+ channels within the small area of the patch membrane were blocked by intrapipette TMP in our single channel measurements, the entire apical surface of A6 cells was exposed to TMP in I_{sc} measurements [1]. Therefore, total apical Na^+ entry into the A6 cells and thus, feedback inhibition would be much less in previous I_{sc} experiments than in our cell-attached patch experiments.

Conclusions

We have shown that apical TMP, at concentrations found clinically in the urine, directly and reversibly blocks distal nephron Na^+ channels in a manner similar to K^+-sparing diuretics such as amiloride. The result is a decrease in the electrochemical driving force for renal tubular K^+ secretion and likely explains the reduced K^+ excretion and increased serum potassium associated with HIV-infected patients receiving high dose TMP in the absence of adrenal insufficiency, hyporeninemic hypoaldosteronism, renal failure or tubulointerstitial damage.

Acknowledgments

L.E.S. was supported by NIH Training Grant T32-DK07656 and NIH Grant R01-DK37963, T.R.K. by VA Merit Review Award and AHA Established Investigator Award, and B.N.L. by NIH Clinical Investigator Award K08-DK02111 and VA Merit Review Award.

Portions of this work have been presented at the Experimental Biology '93 Meeting [24] and American Society of Nephrology Annual Meeting, November '93 [25].

The authors thank Dr. Pedro C. Fernandez for his astute clinical observations that served as the catalyst for these studies. We are also indebted to Elisabeth E. Seal for her technical assistance in the preparation of the A6 cell cultures.

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